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Development and Validation of RP-LC Method for the Determination of Cinnarizine/Piracetam and Cinnarizine/Heptaminol Acefyllinate in Presence of Cinnarizine Reported Degradation Products

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Abstract: Specific stability indicating reverse-phase liquid chromatography (RP-LC) assay method (SIAM) was developed for the determination of cinnarizine (Cinn)/piracetam (Pira) and cinnarizine (Cinn)/heptaminol acefyllinate (Hept) in the presence of the reported degradation products of Cinn. A C₁₈ column and gradient mobile phase was applied for good resolution of all peaks. The detection was achieved at 210 nm and 254 nm for Cinn/Pira and Cinn/Hept, respectively. The responses were linear over concentration ranges of 20–200, 20–1000 and 25–1000 µgmL⁻¹ for Cinn, Pira, and Hept respectively. The proposed method was validated for linearity, accuracy, repeatability, intermediate precision, and robustness via statistical analysis of the data. The method was shown to be precise, accurate, reproducible, sensitive, and selective for the analysis of Cinn/Pira and Cinn/Hept in laboratory prepared mixtures and in pharmaceutical formulations.

Keywords: cinnarizine, piracetam, heptaminol acefyllinate, stability indicating assay method (SIAM), reverse-phase liquid chromatography (RP-LC)

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Introduction

Cinnarizine (Cinn; Fig. 1a) is a piperazine derivative with H₁ blocking (antihistaminic) and calcium channel blocking activity.^{1,2} Cinn is used for prevention and treatment of nausea and motion sickness.² Piracetam (Pira; Fig. 1b) 2-oxo-1-pyrrolidine acetamide¹ acts on the central nervous system. Pira has neuroprotective properties and improves neuroplasticity. At a vascular level, Pira appears to reduce erythrocyte adhesion to vascular endothelium, hinders vasospasm, and facilitates microcirculation.² Heptaminol acefyllinate (Hept; Fig. 1c) 6-amino-2-methylheptan-2-ol salt of theophyllin-7-ylacetic acid is a peripheral vasodilator.²

Several analytical methods have been reported for the determination of Cinn, Pira and Hept. Cinn samples, for example, have been analyzed by non-aqueous titration³ and both colorimetric and derivative spectrometric methods have been reported for the determination of Cinn.⁴⁻⁷ Several liquid chromatographic (LC) methods have also been described in the literature for the determination of Cinn, such as LC with UV detection,⁸⁻¹² LC with mass spectrometric detection (MS),^{13,14} and LC with fluorimetric detection.¹⁵ Additionally, electrophoresis with UV detection has also been used for the determination of Cinn.¹⁶ Second-derivative synchronous fluorescence spectrometry was used for the determination of Cinn in pharmaceutical formulation¹⁷ and thin-layer chromatographic (TLC)-densitometric method was applied for the simultaneous determination of Cinn and Pira in pharmaceutical preparations.⁹

For the determination of Pira in pharmaceutical formulations several analytical methods have been reported including spectrometry,^{18,19} LC with UV detection,^{3,20-22} and LC with evaporative light scattering detection.²³ Pira was determined in rat plasma by LC with MS detection.²⁴ Furthermore, electrophoresis with UV detection,²⁵ GC,²⁶ and TLC-densitometric methods have also been used for the determination of Pira.^{27,28}

Only a few analytical methods have been reported for the determination of Hept, such as LC with UV detection and spectrometric methods. The described methods have the advantage of simultaneous determination of Cinn and Hept.²⁹

The aim of the present study is to develop and validate an accurate and reproducible method for the simultaneous determination of Cinn/Pira and Cinn/Hept in the presence of the reported degradation products of Cinn, namely benzophenone, benzhydrol, dibenzhydrol ether, and N-cinnamyl piperazine.³⁰ While the limited number of the reported LC methods with UV detection were capable of simultaneous determination of Cinn in its mixture with Pira⁹ or Hep,²⁹ none of these methods have the ability to separate the degradation products of Cinn.

In conclusion, the previously published analytical techniques do not have the advantage of determining Cinn, Pira, and Hept in the presence of reported degradation products of Cinn.

Experimental

Instrumentation

The high-performance liquid chromatography (HPLC) system consisted of a Hewlett Packard, 1100 series (Waldbronn, Germany), diode array detector, and manual injector with 20 μ L loop using Hypersil BDS C₁₈ column (5 μ m, 200 \times 4.6 mm) (Thermo, Bellefonte, PA, USA). The system was equipped with a UV-visible detector.

Reagents and reference samples

Cinn and Pira standards of pharmaceutical grades were supplied from Misr, pharmaceutical company (Cairo-Egypt), while Hept standards were supplied from Memphis pharmaceutical company (Cairo-Egypt). The percent purity of these compounds were 99.54% \pm 1.18% and 100.56% \pm 1.17%, for Cinn and Pira, respectively, according to reference method

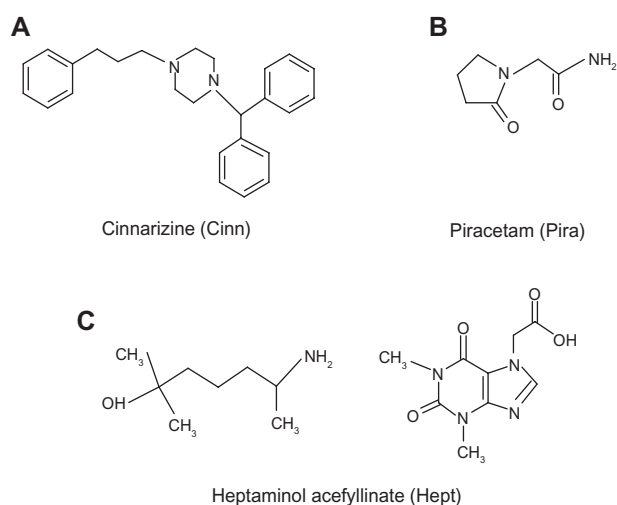


Figure 1. Chemical structure of Cinn (A), Pira (B), and Hept (C).



BP,³ and 100.00% ± 1.53% for Hept, according to the company method.

Degradation product of Cinn was benzophenone (Prolabo, France).

Benzhydrol, dibenzhydrol ether, and N-cinnamylpiperazine were prepared using the published procedure.^{30–33}

Cinnaretam tablets of batch number 805072 contained 25 and 400 mg Cinn and Pira/tablets, respectively.

Surpetil tablets of batch number 23009 contained 20 and 200 mg Cinn and Hept/tablets, respectively.

All solvents used were of LC grade and all other reagents were of analytical grade. Potassium dihydrogen orthophosphate and sodium hydroxide were obtained from Merck [Darmstadt, Germany]. Methanol and acetonitrile were obtained from Lab Scan Analytical Sciences [New Jersey, USA]. Phosphate buffer was prepared by dissolving 6.8 g potassium dihydrogen orthophosphate in 900 mL water, adjusting pH with 0.1 M sodium hydroxide and diluting to 1 L with water.

Four stock solutions were prepared: stock solution 1 contained 0.2 mgmL⁻¹ Cinn in acetonitrile; stock solution 2 contained 1 mgmL⁻¹ Pira in acetonitrile; stock solution 3 contained 2 mgmL⁻¹ Hept in acetonitrile; and stock solution 4 contained 0.5 mgmL⁻¹ of each of the degradation products in acetonitrile.

Chromatographic conditions

Chromatographic separation was achieved on a Hypersil BDS C₁₈ column (5 μm, 200 × 4.6 mm) (Thermo, Bellefonte, PA, USA), with gradient elution using mobile phase A (acetonitrile: phosphate buffer (pH 6) (60:40 v/v)) and mobile phase B (acetonitrile: phosphate buffer (pH 6) (80:20 v/v)). The gradient programming is shown in Table 1. The detection was done at 210 nm and 254 nm for Cinn/Pira and Cinn/Hept,

Table 1. LC mobile phase program.

Time (in minutes)	Mobile phase A	Mobile phase B
0	100%	0%
6	100%	0%
6.1	0%	100%
21	0%	100%
21.1	100%	0%
26	100%	0%

respectively. The mobile phase was pumped through the column at a flow rate of 1 mLmin⁻¹. Separation was done at ambient temperature and the run time was 26 min.

Sample preparation

Twenty cinnaretam and sureptil tablets were ground and thoroughly mixed. An amount equivalent to 25/400 and 20/200 mg of Cinn/Pira and Cinn/Hept, respectively, were accurately weighed, transferred to separate 100 mL volumetric flask, and dissolved in 20 mL of acetonitrile. The solutions were sonicated for 10 min and then cooled. The solutions were diluted to volume with acetonitrile and filtered.

Procedure

Linearity and repeatability

Accurately measured volumes of stock solutions 1, 2, and 3 equivalent to 0.2–2, 0.2–10, and 0.25–10 mg Cinn, Pira and Hept, respectively, were transferred into three separate series of 10 mL volumetric flasks and the solutions were completed to volume with acetonitrile. Each solution (20 μL) was injected in triplicates. A calibration plot for each drug was obtained by plotting the area under the peak against drug concentration 20–200, 20–1000, and 25–1000 μgmL⁻¹ for Cinn, Pira and Hept, respectively, and regression equations were computed.

Assay of sample preparation

The procedure previously described was repeated using a volume of standard solution equivalent to 0.2–2, 2–10, and 2–10 mg of Cinn, Pira and Hept, respectively, and the concentration of each drug was determined by the use of its corresponding regression equation.

Assay of drugs in laboratory prepared mixtures

The procedure described previously was repeated using a mixture of standard solutions equivalent to 0.2–2 mg Cinn, 0.2%–50% degradation products, and 2–5 mg of Pira and Hept. The concentration of each drug was determined by the use of its corresponding calibration equation.

Results and Discussion

The primary objective of the proposed LC method was the simultaneous determination of a mixture

of Cinn/Pira and Cinn/Hept in the presence of the reported degradation products of Cinn. Degradation products of Cinn were:

1. Benzophenone (Prolabo, France).
2. Benzohydrol was prepared using the published procedure.³¹ The purity was checked by m.p. and comparison of its IR spectrum (KBr) with that of benzophenone, which showed the appearance of O-H stretching band at 3300 cm^{-1} and disappearance of C=O stretching band at 1640 cm^{-1} .
3. Dibenzohydrol ether was prepared from benzhydrol using the published procedure.³² The purity was checked by m.p. and comparison of its IR spectrum (KBr) with that of benzophenone and benzhydrol. It showed the absence of both C=O stretching and O-H stretching bands at 1640 cm^{-1} and 3300 cm^{-1} , respectively.
4. N-Cinnamylpiperazine was reported in the literature as a patent.³³ However a pure sample was prepared and its structure was confirmed by MS and $^1\text{H-NMR}$. The MS chart revealed m/z 202 molecular ion, m/z 117 base peak and m/z 85.

The proposed method gave sharp, well resolved peaks of both drugs under investigation and the four known degradation products (Figs. 2 and 3). None of the published chromatographic methods were capable of achieving the resolution of the six aforementioned components.

Method development

To optimize the LC assay parameters, the mobile phase composition was studied. A satisfactory separation of all peaks was obtained using gradient programming of mobile phase (Table 1). Changing the proportion of the organic to aqueous ratio in mobile phases A and B by 10% achieved good resolution, but still prolonged retention time. High volume ratio of acetonitrile showed good separation of Cinn, however inadequate separation for Pira and Hept and prolonged retention time for other compounds. On the other side, lower volume ratio of acetonitrile showed adequate separation for Pira and Hept, and prolonged elution time for Cinn. High volume ratio of acetonitrile is still needed for the separation of N-Cinnamylpiperazine, which could not be separated at lower organic to aqueous ratio. This accounts for the composition of the mobile phase B (80% acetonitrile) and the need for gradient programming to achieve separation for all 6 compounds under investigation. Different C_{18} columns were tried, such as X-Terra ($5\text{ }\mu\text{m}$, $250 \times 4.6\text{ mm}$), Thermo Hypersil ODS ($5\text{ }\mu\text{m}$, $200 \times 4.6\text{ mm}$), and μ -Bondapak ($5\text{ }\mu\text{m}$, $300 \times 4.6\text{ mm}$). All columns achieved good resolution of all peaks under investigation. The advantage of Thermo Hypersil BDS C_{18} ($5\text{ }\mu\text{m}$, $200 \times 4.6\text{ mm}$), however, was the less run time required for elution of the 6 studied peaks. The system suitability test results of the developed method are presented in Table 5.

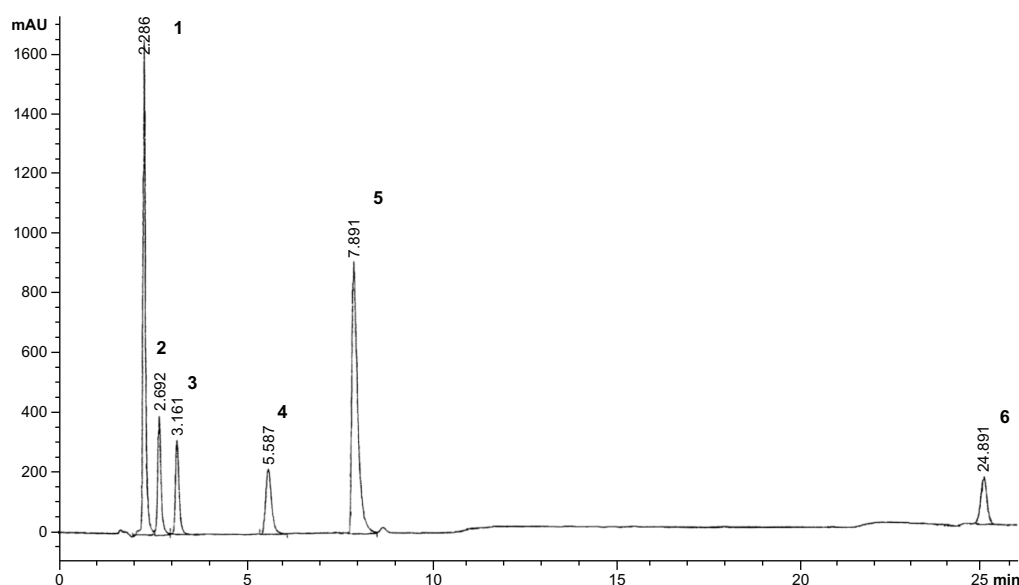


Figure 2. A typical LC chromatogram of laboratory prepared mixture of (5) Cinn & its degradation products ((2) Benzhydrol (3) Dibenzohydrol ether (4) Benzophenone (6) N-Cinnamylpiperazine) in presence of (1) Pira.

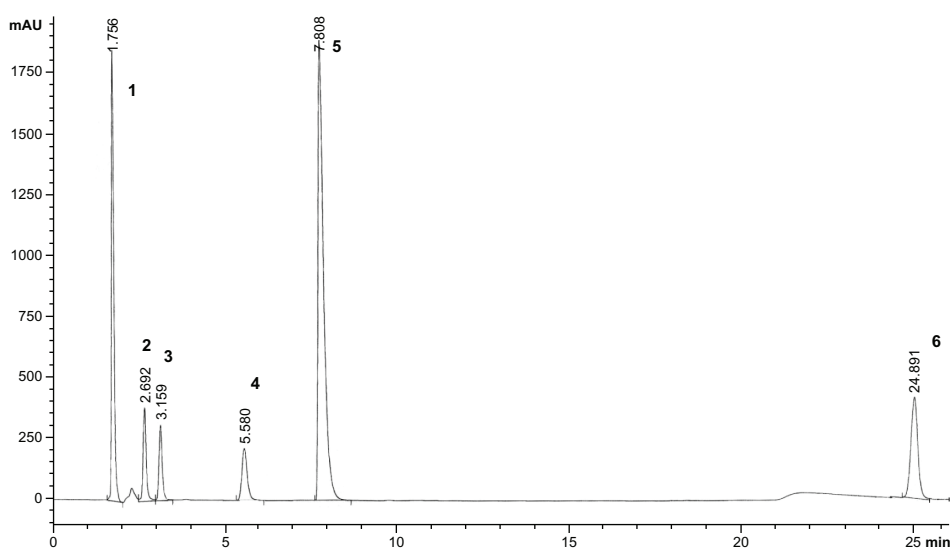


Figure 3. A typical LC chromatogram of laboratory prepared mixture of (5) Cinn & its degradation products ((2) Benzhydrol (3) Dibenzohydrol ether (4) Benzophenone (6) N-Cinnamylpiperazine) in presence of (1) Hept.

Analysis of pharmaceutical product

The proposed LC method was applied to the determination of Cinn/Pira and Cinn/Hept in tablets. Satisfactory results were obtained for Cinn/Pira and Cinn/Hept in good agreement with the labeled claimed Tables 2 and 3.

Method validation

Linearity

Linearity was studied for Cinn/Pira and Cinn/Hept by the proposed LC method. Linear relationship was

obtained between area under the peak and each drug concentration. First order equation was used for the calibration. The analytical data for the regression equation are summarized in Tables 2 and 3.

Range

The calibration ranges were established through consideration of the practical range necessary, according to each compound concentration present in pharmaceutical product, to give accurate, precise, and linear results. Wide calibration ranges were obtained for

Table 2. Validation data for analysis of Cinn and Pira using LC method.

	Cinn	Pira
Linear range ($\mu\text{g mL}^{-1}$)	20–200	20–1000
Regression equation	Area = 45.795X + 1.2639	Area = 18.814X + 866.7
Regression coefficient	0.9999	0.9985
LOQ (%) ¹	0.2	0.1
LOD (%) ¹	0.05	0.05
Concentration ($\mu\text{g mL}^{-1}$)	20 50 100	150 250 500
Repeatability (n = 3)	0.65 0.45 0.31	0.333 0.273 0.042
Intermediate precision (n = 9)	0.82 0.64 0.92	1.386 0.398 0.590
Standard error of estimation	69.42	56.9
S_a	31.65	16.4
S_b	0.42	0.07
Results from sample analysis		
1. Drug in bulk ²	100.75 ± 1.38	100.01 ± 1.03
2. Drug in dosage form ²	106.12 ± 0.80	97.34 ± 0.58
3. Drug added ²	100.14 ± 1.05	100.38 ± 0.65
4. Drug recovery in presence of degradation products ² (0.2%–50%)	101.24 ± 2.10	100.27 ± 1.696

Notes: ¹100% Corresponds to 200 & 1000 $\mu\text{g mL}^{-1}$ injected for Cinn & Pira, respectively; S_a , standard error of slope; S_b , standard error of intercept. ²Results (% recovery* ± RSD) (*average of three experiments).

**Table 3.** Validation data for analysis of Cinn and Hept using LC method.

	Cinn	Hept
Linear range ($\mu\text{g mL}^{-1}$)	20–200	25–1000
Regression equation	Area = 45.795X + 1.2638	Area = 17.76 + 418.31
Regression coefficient	0.9999	0.9985
LOQ(%) ¹	0.2	0.2
LOD(%) ¹	0.05	0.05
Concentration ($\mu\text{g mL}^{-1}$)	20 50 100	200 500 1000
Repeatability (n = 3)	0.65 0.45 0.31	0.83 0.72 1.24
Intermediate precision (n = 9)	0.82 0.64 0.92	1.02 0.94 1.62
Standard error of the estimation	69.42	64.64
S _a	31.65	20.98
S _b	0.42	0.07
Results from samples analyses		
1. Drugs in bulk ²	100.75 ± 1.38	100.00 ± 1.53
2. Drugs in dosage form ²	98.96 ± 1.02	95.47 ± 0.86
3. Drugs added ²	99.79 ± 1.15	99.74 ± 1.21
4. Drugs recovery in Laboratory prepared mixture ² (0.2%–50%)	101.24 ± 2.10	98.24 ± 1.70

Notes: ¹100% Corresponds to 200 & 1000 $\mu\text{g mL}^{-1}$ injected for Cinn & Hept, respectively; S_a, standard error of slope; S_b, standard error of intercept. ²Results (% recovery* ± RSD) (*average of three experiments).

both Cinn/Pira and Cinn/Hept. The calibration range of each compound is given in Tables 2 and 3.

Accuracy and precision

The accuracy of the results was calculated as percentage recovery from six different concentrations of the drugs in bulk powder analyzed by the proposed method. The obtained results were statistically compared with those obtained by the BP 2012³ for Cinn and Pira, and by the company method for Hept, using student's *t*-test and F ratio. The obtained results are presented in Table 4 and were indicative of the accuracy of the proposed method. Precision was assessed by repeatability and intermediate precision. Repeatability was measured by analysis of three different concentrations of the test solution,

while intermediate precision was assessed by the determining the RSD of the concentrations of each compound on three different days. Repeatability was further assessed by determining the concentration of each compound in the mentioned test solution after being spiked with the degradation product mixture. Results were summarized in Tables 2 and 3.

Specificity

Specificity is the ability of an analytical method to measure analyte response in the presence of interferences. Specificity of the proposed method and its good potential to determine both Cinn/Pira and Cinn/Hept in the presence of the four reported degradation products was assessed by the determination of both drugs in laboratory prepared mixture (Figs. 2 and 3). It was

Table 4. Results from the use of the proposed LC methods and reference methods for the analysis of Cinn/Pira and Cinn/Hept in Bulk material.

	Recovery of Cinn		Recovery of Pira		Recovery of Hept	
	Reference method ^[3]	LC method	Reference method ^[3]	LC method	Reference method ^a	LC method
Mean ± RSD	99.54 ± 1.18	100.75 ± 1.38	100.56 ± 1.17	100.01 ± 1.03	99.94 ± 1.25	100.00 ± 1.53
F (6.39) ^b	–	1.42	–	1.28	–	1.50
t (2.31) ^b	–	1.49	–	0.79	–	0.68
Variance	1.36	1.93	1.37	1.07	1.56	2.34
SD	1.17	1.39	1.17	1.04	1.25	1.53

Notes: ^aManufacturer's method. ^bFigures in parenthesis represents corresponding tabulated values for *t* and *F* at *P* = 0.05.



also proven by the ability of the proposed method to determine Cinn/Pira and Cinn/Hept in sample solution, without interference of excipients. Results were summarized in Tables 2 and 3.

Robustness

The robustness of the proposed LC method was assessed by the ability to remain unaffected by small changes in experimental conditions (flow rate, mobile phase, and temperature). Changing flow rate by ± 0.2 , temperature by ± 5 °C, and fine changes of aqueous to organic ratio by 10% did not affect the resolution of all peaks, proof of the method robustness.

Limits of quantification and limit of detection

The limit of quantification (LOQ) and detection (LOD) were defined as the concentration for which the signal-to-noise ratios (S/N) were ten and three, respectively. The values obtained, which were averages from six replicates, are given in Tables 2 and 3.

Chromatographic performance parameters

System suitability parameters were calculated according to USP 2011.³⁴

Resolution factor (R)

Resolution is a measure of the degree of separation between adjacent peaks. A value of 1.5 for resolution implies a complete separation of two compounds. In the present work, the resolution value for the separation of each compound was greater than 1.5 and is represented in Table 5.

Selectivity factor (α)

Selectivity parameter is a measure of separation of two compounds in the sample under given conditions. The calculated selectivity parameter for separation of each compound is represented in Table 5.

Capacity factor (k')

Capacity factor (retention factor) is a measure of the retention time of a compound in the sample with a given combination of mobile phase and column. Calculated k' values are represented in Table 5.

Tailing factor (T)

Tailing factor refers to peak asymmetry. Many chromatographic peaks do not appear in the shape of normal Gaussian distribution. Therefore, tailing factor should be calculated. A tailing factor of 1 refers to a symmetric peak. The calculated values for each compound is in the acceptable range and is represented in Table 5.

Number of theoretical plates (Column efficiency) (N)

In particular separation, column efficiency refers to the performance of the stationary phase. It refers to how well the column is packed. The N values for each compound are represented in Table 5.

Conclusion

The proposed RP-LC method provides accurate, reproducible, and specific stability indicating assay method (SIAM), where the method is able to determine Cinn in presence of its reported degradation products, excipients, additives, Pira, and Hept. No methods have previously been reported for the determination of Cinn in presence of its degradation products, Pira and Hept. The results obtained from this method were compared with those obtained by the official methods and show no significance difference. The method can be applied for the determination of both drugs in pharmaceutical dosage form in routine work and quality control laboratories.

Author Contributions

Conceived and designed the experiments: OMH, NHZ, MAM. Analyzed the data: OMH, NHZ, MAM. Wrote the first draft of the manuscript: OMH. Contributed to the writing of the manuscript: OMH, NHZ, MAM. Agree with manuscript results and conclusion: OMH,

Table 5. System suitability.

	For Cinn/Pira method		For Cinn/Hept method	
	Cinn	Pira	Cinn	Hept
Resolution factor (R)	8.65	3.06	6.92	8.4
Selectivity factor (α)	1.41	1.18	1.5	1.4
Retention factor (k')	3.777	0.384	6.1	0.6
Tailing factor (T)	0.49	0.72	0.43	0.72
Number of theoretical plates (N)	15106	5209	3599	14363
Retention time (t_r)	7.891	2.286	7.808	1.756



NHZ, MAM. Jointly developed the structure and argument for the paper: OMH, NHZ, MAM. Made critical revisions and approved final version. OMH, NHZ, MAM. All authors reviewed and approved of the final manuscript.

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Competing Interests

Author(s) disclose no potential conflicts of interest.

Disclosures and Ethics

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

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